Indian Journal of Medical Microbiology, (2015) 33(3): 374-377

Original Article

Detection of clarithromycin-resistant *Helicobacter pylori* strains in a dyspeptic patient population in Sri Lanka by polymerase chain reaction-restriction fragment length polymorphism

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Abstract

Aim: The aim of this study was to investigate the proportion of common clarithromycin-resistant mutation types present in the 23S ribosomal ribonucleic acid (rRNA) gene of *H. pylori* strains in Sri Lanka. **Settings and Design:** The study was a cross-sectional, descriptive study where 76 dyspeptic patients who were required to undergo endoscopy examination were included. The study was carried out at a Teaching Hospital in Sri Lanka. **Subjects and Methods:** In-house urease test and polymerase chain reaction (PCR) amplification of the glmM gene of *H. pylori* was performed to confirm the *H. pylori* infection. Analysis of point mutations in 23S rRNA gene strains were performed by PCR-restriction fragment length polymorphism (RFLP). **Results:** Of the 16 urease-positive biopsies, 94% (n = 15) were positive by PCR using the glmM primer. All *H. pylori* strains yeilded a point mutation at A2142G site of the 23S rRNA gene, while A2143G mutation was not detected. **Conclusions:** For the first time in Sri Lanka, we reported predominance of A2142G point mutation associated with claritromycin resistance of *H. pylori* in a Sri Lankan population.

Key words: *Clarithromycin, Helicobacter pylori, mutation, restriction fragment length polymorphism, 23S ribosomal ribonucleic acid gene*

Introduction

The global prevalence of *Helicobacter pylori* infection is more than 50%, and it may vary significantly within and between countries.[1,2] *H. pylori* prevalence in Sri Lanka has been reported to be as high as 70%.[3] Its severe pathological manifestations and difficulty in culture, results in the use of empirical antibiotics for treatment. Thus, it is of paramount importance to determine the antibiotic resistance of *H. pylori* in Sri Lanka.

Clarithromycin is one of the key antibiotics used for the treatment[4] of *H. pylori*. Clarithromycin resistance is increasing worldwide (the primary resistance rate is 9.9% globally)[5], and is the main factor reducing the efficacy of *H. pylori* eradication.[6] There are no reports of the antibiotic sensitivity patterns of *H. pylori* in Sri Lanka based on phenotypic or genotypic analysis up to now. Clarithromycin resistance is detected by *in vitro* antibiotic susceptibility tests (disk diffusion, agar dilution and Epsilometer-test methods), but are not easy to perform for *H. pylori* since it requires isolation of this fastidious bacterium.[7] Hence polymerase chain reaction (PCR)-based molecular methods have been performed to determine the clarithromycin resistance of *H. pylori* strains.[7,8]

Studies have shown a geographic and ethnic variation[4,9] in the presence of clarithromycin resistance mutations and hence the data available for other countries cannot be directly applied for the local setting. Therefore, the proportion of clarithromycin resistance of *H. pylori* was investigated in this study. To our knowledge, this is the first study conducted in Sri Lanka to detect antibiotic resistance of *H. pylori*.

Subjects and Methods

The study was a cross-sectional, descriptive study where 76 dyspeptic patients who were required to undergo endoscopy examination were included. The study was carried out in a tertiary care teaching hospital in Sri Lanka which caters to several provinces in Sri Lanka.

The study was conducted between June 2013 to January 2014 where two biopsies from each patient was

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Received:19-03-2014
Accepted: 21-01-2015

Access this article online

Quick Response Code:

Website: www.ijmm.org

PMID: ***

DOI: 10.4103/0255-0857.158557

obtained (Female-43 and Male-33) during the endoscopy procedure from antrum/ulcer lesion into normal saline. Laborotory investigations were done at the Department of Microbiology and the Department of Pathology, in a state university, Sri Lanka.

The proportion of *H. pylori* in gastric biopsies were determined by using an in-house biopsy urease test.[10] The samples which were positive for the in-house urease test within 12 hours were used for deoxyribonucleic acid (DNA) extraction by QIAamp DNA mini kit (Qiagen, Germany) following the manufacturer's instructions. The extracted DNA was amplified by PCR using primers targetting glmM (*ureC*) gene[11] for identification of *H. pylori* and fragment of the peptidyltransferase region of the 23S ribosomal ribonucleic acid (rRNA) gene[8] of *H. pylori* for mutation detection [Table 1]. The urease test-positive samples were further confirmed for *H. pylori* infection by histopathology (Haematoxylin and Eosin staining) prior to Helicobacter-specific PCR.

Clarithromycin resistance of *H. pylori* was determined by PCR-restriction fragment length polymerphism (RFLP). All PCRs were performed in 0.2-ml tubes using a Flexigene thermal cycler (version 31.04). The 50 µl PCR reaction mixture consisted of PCR buffer (5 µl) supplemented with 2 mM $MgCl_2$ (Go Taq Flexi DNA polymerase kit; Promega), 0.2 mM each dATP, dCTP, dGTP and dTTP (1 µl; Promega), 0.5 µM each primer (Integrated DNA Technologies), 1.25U of Go Taq Flexi DNA polymerase (promega) and 2 µl of nucleic acid (~20 ng). Optimized PCR conditions for glmM gene amplified were denaturation at 93°C for 5 min, PCR cycle at 93°C for 1 min, 55°C for 1 min and 72°C for 1 min, PCR was performed for 40 cycles with a final extension at 72°C for 5 min. PCR parameters for 23S rRNA included an initial 5 min denaturation at 94°C. The PCR cycles consisted of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were visualized in a 1.5% agarose gel and resulted in a 294 base pair product for glmM gene and 1400 bp product for 23SrRNA gene [Figure 1].

Restriction enzymes *Mbo*II (Thermo Scientific, USA) and *Bsa* I (New England Biolabs, Mass, USA) were used in separate reactions to digest the 23SrRNA PCR products. The amplicon was digested with 2.5U of *Mbo* II at 37°C for 16 hours and the restriction products were analyzed on a 2% agarose gel. Mutation at A2142G was detected by the cleavage of the 1400bp amplicon resulting in a single 700bp band in the gel. In the wild type, no digestion was detected.

Mutation A2143G was detected by digesting the amplicon with *Bsa* 1 restriction enzyme in the following conditions. *Bsa* I (2.5U) was digested with the amplicon of 23S rRNA gene at 37°C for 16 hours. Mutation A2143G was detected by the cleavage of the 1400 bp amplicon resulting in three bands at 700 bp, 400 bp and 300 bp in the gel. In the wild type, restriction digestion resulted in two bands at 1000 bp and 400 bp in the gel [Figure 2].

Statistical analyses were done with Chi-square test using the Statistical Package of Social Sciences (SPSS) (version 16; SPSS, Inc., Chicago, Illinois, USA).

Ethical approval was granted by ethical review committee, at the state university (No: 723/13).

Results

Among the dyspeptic patients, 43 (57%) were female while 33 (43%) were male. Median age was 50 years. Endoscopic examination identified the disease as antral gastritis in 53/76 (70%), gastric ulcer disease in 9/76 (12%), pangastritis in 8/76 (10%) and duodinitis in 6/76 (8%) patients. Ten 10/53 (19%) patients who were identified as suffering from antral gastritis was positive for *H. pylori* by in-house campylobacter like organism (CLO) test while 1/10 (11%) among gastric ulcer patients, 3/8 (38%) of pangastritis patients and 2/6 (33%) among duodinitis patients were also positive for *H. pylori* by this test. These samples were positive for *H. pylori* by the in-house CLO test with in 12 hours of incubation. Of the 16 urease-positive

Figure 1: Product from polymerase chain reaction (PCR) amplification of 23S ribosomal ribonucleic acid (rRNA) gene of *H.pylori* Lane M:1,000bp ladder, Lane1-4: Positive controls, Lane 5: Negative control, Lane 6–8, 11–14: Amplicon for 23S rRNA gene from patient's biopsy samples, Lane 9,10: No amplicon

Table 1: Oligonucleotide sequences used in this study

Target	Primer	DNA sequence (5'-3')	Position	Amplicon size	Reference
glmM gene	glmM1-F	AAG CTT TTA GGG GTG TTA GGG GTT T	784-809	294	[11]
	glmM2-R	AAG CTT ACT TTC TAA CAC TAA CGC	1053-1077		
23S rRNA	Cla18	AGTCGGGACCTAAGGCGAG	1341-1359	1400	[8]
	Cla21	TTCCCGCTTAGATGCTTTCAG	2764-2744		

Table 2: The relationship between presence of *H. pylori* with patient's gender, age, ethnicity, family dimension and type of consumed water

Variable	Positive PCR (%)	Negative PCR (%)	*P* value	Fisher's exact test
Gender				
Female	10 (67)	33 (54)	0.379	0.562
Male	5 (33)	28 (46)		
Age				
<50	9 (60)	28 (46)	0.328	0.395
>=50	6 (40)	33 (54)		
Ethnicity				
Sinhalese	11 (73)	57 (93)	0.023	0.044
Tamils and others	4 (27)	4 (7)		
Family dimension				
<3	2 (13)	5 (8)	0.416	-
3-5	10 (67)	50 (82)		
>5	3 (20)	6 (10)		
Type of water consumed				
Tap water	8 (53)	38 (62)	0.525	0.565
Well water	7 (47)	23 (38)		

PCR: Polymerase chain reaction

biopsies, 15 were confirmed by PCR and histopathology. Further all *glmM* PCR-positive samples were also positive by PCR using primers targeting the 23S rRNA gene. Mutation analysis using restriction enzyme *Mbo* II resulted in detection of A2142G point mutations of the 23S rRNA gene in all the PCR-positive samples (100%). The A2143G mutation was not present in the studied population.

H. pylori infection was more common among females 10/15 (67%) and in the age-group of < 50, 9/15 (60%). A high frequency of infection was also observed among Sinhalese, 11/15 (73%), and there was a statistically significant relationship between ethnicity and presence of *H. pylori* (P = 0.023). The highest frequency of *H. pylori* infection was noted in families with 3 to 5 members [n = 10/15 (67%)]. However, no association was observed between presence of *H. pylori* with patient age, gender, family dimension and type of consumed water [Table 2].

Discussion

In Sri Lanka, clinically suspected *H. pylori* infections are treated with a proton pump inhibitor combined with two antibiotics which included clarithryomycin as a compulsory drug. Unfortunately, this treatment is not tailor made to Sri Lanka since screening for clarithyromyin resistance is not done. Some of the other countries screen the patients for clarithyromyin resistance to *H. pylori* prior to treatment.

The rate of A2142G mutation associated with resistance to clarithromycin was found to be very high in

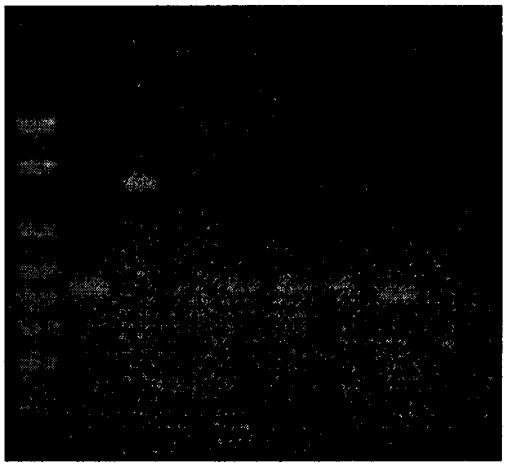

Figure 2: Detection of the A2142G mutation in 23S ribosomal ribonucleic acid (rRNA) gene by *Mbo* II digestion of polymerase chain reaction (PCR) products. Lane M: 100bp ladder, Lane 1 and 2: Positive (700bp) and negative control (1400 bp), Lane 3-7: Digested PCR products of clinical specimens.All had A2142G mutation

Sri Lanka. The proportion of *H. pylori* infection among the dyspeptic patients was found to be decreasing (20%) which is in line with the current global trend of decreasing prevalence.[12,13] However, in Sri Lanka, the finding of high A2142G mutation is alarming. Published studies showed that although A2142G mutation is present it sometimes may not be phenotypically expressed.[14] The eradication of *H. pylori* from patients following treatment is a good indicator to detect the expression of clarithromycin resistance gene[15] and which can be practically done by detecting *H. pylori* antigen in stool and will be done in the future. Therefore, currently, we cannot conclude the efficacy of clarithromycin in the treatment of *H. pylori* in Sri Lanka.

The countries with low clarithromycin resistance are recommended the standard clarithromycin containing triple therapy, whereas the other countries use a tailor made treatment for a better outcome. According to some investigators, A2142G mutation site was less common when compared to A2143G mutation.[16] Whereas in some countries, both A2142G and A2143G mutations of *H. pylori* were found to occur in equal frequency.[17] Our findings were in line with the results of other developing countries such as Iran and Korea where they have encountered A2142G mutation as the most common mutation.[18,19] In our study, A2143G mutation was not detected. Further presence of *H. pylori* was significantly associated with ethnicity which may be due to food habits and cultural practices. Simultaneously, clarithromycin resistance was also significantly associated to ethnicity. There may be certain factors associated to ethic groups which may predispose to development of antibiotic resistance. The clinical significance of this matter cannot be discussed further

because phenotypic expressions of these mutations were not detected as yet.

Different studies done in different geographic populations have showed high clarithromycin resistance with either one of the A2142G/A2143G mutations, thus is not a finding specific to this study. Raymond *et al.*[20] in 2007 had found a 90% prevalence of A2143G mutation, while Abdollahi *et al.*,[18] in 2011 had shown a 55% prevalence of A2142G mutation, whereas Kim KS *et al.*[19] in 2002 had reported 100% mutation in A2143G or T2182C sites in Korea. Studies have shown that phenotypic and genotypic expressions of clarithromycin resistance do not correlate.[14] In a study by Vincenzo De Francesco *et al.*,[14] it was shown that clarithromycin phenotypic resistance was significantly lower as compared with genotypic resistance (18.4% versus 37.6%).

The current results indicate the importance of determination of the eradication efficacy of *H. pylori* following clarithromycin treatment which can give an insight regarding the phenotypical expression of the A2142G mutation in Sri Lanka. If the mutation is phenotypically expressed screening for resistance prior to initiation of treatment may be required in the future.

Conclusion

This study documented for the first time a high proportion of A2142G mutation associated with clarithromycin resistance in *H. pylori* among dyspeptic patients in Sri Lanka.

Acknowledgement

We thank State Pharmaceuticals Corporation (SPC) of Sri Lanka for financial support. Also, we would like to thank to nursing staff at Endoscopy unit of Teaching Hospital, Sri Lanka.

References

1. Khalifa MM, Sharaf RR, Aziz RK. *Helicobacter pylori*: A poor man's gut pathogen? Gut Pathogens 2010;2:2.
2. Liu Z, Shen J, Zhang L, Shen L, Li Q, Zhang B, *et al.* Prevalence of A2143G mutation of H. pylori-23SrRNA in Chinese subjects with and without clarithromycin use history. BMC Microbiol 2008;8:81.
3. Fernando N, Holton J, Vaira D, Desilva M, Fernando D. Prevalence of *Helicobacter pylori* in Sri Lanka as determined by PCR. J Clin Microbiol 2002;40:2675-7.
4. Cui R, Zhou L. *Helicobacter pylori* infection: An overview in 2013, focus on therapy. Chin Med J 2014;127:568-73.
5. Megraud F. *H. pylori* antibiotic resistance: Prevalence, importance, and advances in testing. Gut 2004;53:1374-84.
6. Giorgio F, Principi M, De Francesco V, Zullo A, Losurdo G, Di Leo A, *et al.* Primary clarithromycin resistance to *Helicobacter pylori*: Is this the main reason for triple therapy failure?. World J Gastrointest Pathophysiol 2013;4:43-6.
7. Megraud F, Lehours P. *Helicobacter pylori* detection and antimicrobial susceptibility testing. Clin Microbiol Rev 2007;20:280-322.
8. Versalovic J, Shortridge D, Kibler K, Griffy MV, Beyer J, Tanaka SK, *et al.* Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. Antimicrob Agents Chemother 1996;40:477-80.
9. Wu W, Yang Y, Sun G. Recent insights into antibiotic resistance in *Helicobacter pylori* eradication. Gastroenterol Res Pract 2012;2012:723183.
10. Levin D, Watermeyer G, Mohamed N, Epstein DP, Hlatshwayo SJ, Metz DC. Evaluation of a locally produced rapid urease test for the diagnosis of *Helicobacter pylori* infection. S Afr Med J 2007;97:1281-4.
11. Lu J, Perng C, Shyu R, Chen C, Lou Q, Chong SK, *et al.* Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. J Clin Microbiol 1999;37:772-4.
12. Bureš J, Kopáčová M, Koupil I, Seifert B, Skodová Fendrichová M, Spirková J, *et al.* Significant decrease in prevalence of *Helicobacter pylori* in the Czech Republic. World J Gastroenterol 2012;18:4412-8.
13. Kawakami E, Machado RS, Ogata SK, Langner M. *Helicobacter pylori* infection during a 10-year period in Brazilian children. Arq Gastroenterol 2008;45:147-51.
14. Francesco VD, Zullo A, Ierardi E, Giorgio F, Perna F, Hassan C, *et al.* Phenotypic and genotypic *Helicobacter pylori* clarithromycin resistance and therapeutic outcome: Benefits and limits. J Antimicrob Chemother 2010;65:327-32.
15. Garza-González E, Perez-Perez GI, Maldonado-Garza HJ, Bosques-Padilla FJ. A review of *Helicobacter pylori* diagnosis, treatment, and methods to detect eradication. World J Gastroenterol 2014;20:1438-49.
16. Agudo S, Pérez-pérez G, Alarcón T. Originals rapid detection of clarithromycin resistant *Helicobacter pylori* strains in Spanish patients by polymerase chain reaction-restriction fragment length polymorphism Clinical isolates. Rev Esp Quimiter 2011;24:32-6.
17. Ahmad N. Characterization of clarithromycin resistance in Malaysian isolates of *Helicobacter pylori*. World J Gastroenterol 2009;15:3161-5.
18. Abdollahi H, Savari M, Zahedi MJ, Moghadam SD, Hayatbakhsh Abasi M. Detection of A2142C, A2142G, and A2143G Mutations in 23s rRNA gene conferring resistance to clarithromycin among *Helicobacter pylori* isolates in Kerman, Iran. Iran J Med Sci 2011;36:104-10.
19. Kim KS, Kang JO, Eun CS, Han DS, Choi TY. Mutations in the 23S rRNA Gene of *helicobacter pylori* associated with clarithromycin resistance. J Korean Med Sci 2002;17:599-603.
20. Raymond J, Burucoa C, Pietrini O, Bergeret M, Decoster A, Wann A, *et al.* Clarithromycin resistance in *helicobacter pylori* strains isolated from french children: Prevalence of the different mutations and coexistence of clones harboring two different mutations in the same biopsy. Helicobacter 2007;12:157-63.

How to cite this article: Ubhayawardana NL, Weerasekera MM, Weerasekera D, Samarasinghe K, Gunasekera CP, Fernando N. Detection of clarithromycin-resistant *Helicobacter pylori* strains in a dyspeptic patient population in Sri Lanka by polymerase chain reaction-restriction fragment length polymorphism. Indian J Med Microbiol 2015;33:374-7.

Source of Support: State Pharmaceuticals Corporation (SPC) of Sri Lanka, **Conflict of Interest:** None declared.